Restriction endonucleases digesting DNA in PCR buffer

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Abstract: Six commonly used restriction endonucleases (REs) (Acc I, Ban II, EcoR I, Hind III, Sac I, Sca I) were tested for their ability to directly digest DNA completely in the Polymerase Chain Reaction (PCR) buffers. The results showed that: with the requirement for additional magnesium supplemented as activator, REs, except EcoR I appeared star activity, completely digested unmethylated lambda DNA after overnight incubation in PCR buffer and functioned as equally well as in recommended Restriction Enzyme Buffer provided with each enzyme; all REs tested completely digested PCR products in PCR buffer, it implied digestion of PCR products may often be performed directly in the PCR tube without the requirement for any precipitation or purification steps; and the concentration of $MgCl_2$ from 2.5 mmol·L^{-1} to 10 mmol·L^{-1} did not significantly affect activity of REs in PCR buffer. This simplified method for RE digestion of PCR products could have applications in restriction fragment length polymorphism (RFLP) analysis and single-stranded conformational polymorphism (SSCP) analysis of large PCR products. However, usage of this procedure for cloning applications needs further data.

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Introduction

Restriction endonucleases (RE) digestion is often required to be performed for the downstream applications of the Polymerase Chain Reaction (PCR) products, including restriction fragment length polymorphism (RFLP) analysis (Ganly *et al.* 1991; McDaniel *et al.* 1991; Saiki *et al.* 1986), single-stranded conformational polymorphism (SSCP) analysis of large PCR products (Iwahana *et al.* 1992; Liu *et al.* 1995), and molecular cloning of the amplified DNA (Scharf *et al.* 1986). A common procedure recommended before conducting RE digestion is to purify and recover PCR products with gel electrophoresis and a wide variety of commercial kits and techniques. However, these steps within the common procedure may be time-consuming and expensive, particularly if large numbers of samples are to be analyzed.

The purpose of our study was to determine if REs retained sufficient activity in PCR buffers, and furthermore to allow REs directly added to digest the PCR product in the tube immediately after the amplification step without requirement to purify and recover the PCR product. The results could be composed as a standard protocols for using REs been tested in routine experiments.

Materials and Methods

Unmethylated lambda DNA (Takara) was purchased and used as substrate for RE digestion and template for PCR amplification. EcoR I and Hind III were purchased from NEB, Ban II, Sac I and Sca I were from Takara. *Taq* DNA Polymerase, 10X PCR buffer without $MgCl_2$ and separate 25 mmol·L^{-1} $MgCl_2$ solution were bought from Promega.

Restriction digestion of lambda DNA in PCR buffer

The basal 25 μl of digestion reaction system contained 1μg of Lambda DNA and 10 units of each RE to test for their ability to digest the target DNA in different buffer systems (PCR buffer or RE buffer), different incubation times (1 hour or 15 hours), and various concentration of Mg^{2+}. After 1 hour or 15 hours incubation, all tubes were heated to 65°C for 15 min to de-active RE, then quick-chilled on ice immediately prior to agarose gel electrophoresis (1% agarose gels in 0.5X TBE buffer). Our aims are to determine what basal conditions are required to allow REs to function adequately in PCR Buffer with minimal alteration to the buffer by addition of extra reagents (e.g., Mg^{2+}) and the possible dose-dependent and time-course models of REs activity upon concentrations of extra reagents.

Restriction digestion of PCR products in PCR buffer

A pair of primers (zm1: ATTCTGACTGTAGCTGCTGA and zm2: AATTCATGGACAGTTCGCAC) were designed to amplify about 1.9 kb DNA segment containing recognizing sites of the five REs. PCR reaction was performed in a final volume of 25 μl containing 100 ng lambda DNA, 67 mmol·L^{-1} Tris-HCl (pH 8.9), 50 mmol·L^{-1} KCl, 6 mmol·L^{-1} EDTA, 0.1% Triton® X100, 0.5 mmol·L^{-1} DTT, 2.5 mmol·L^{-1} $MgCl_2$, 200 μmol·L^{-1} dNTPs, 10 pmol of each primer, 1 unit *Taq* DNA polymerase. Amplifications were performed on a PE 2700 thermocycler. The thermocycle was 94°C for 30 s, 53.4°C for 45 s and 72°C for 1 min, for a total of 30 cycles. The initial denaturation and final extension steps were extended to 5 and 10 min, respectively. Immediately after completion of the amplification, the tubes were chilled to 4°C, 10 units of each enzyme known to cut the 2.3 kb fragment and $MgCl_2$ were added into the PCR tubes to test RE activity. The samples were incubated at 37°C for 1 h or 15 h, and finally the digestion was stopped as described above.

All final products were loaded into 1% agarose gel containing 0.5 μg·mL^{-1} Ethidium bromide (EB), with 30 min at 11V/cm. The pictures were photoed and analyzed with the Bio-Rad Quan-

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tity 1D (ver. 4.5) system.

Results

Each reaction was repeated at least three times. Four of the five REs retained their activity and could completely digested Lambda DNA in PCR buffer, as it would be in original reaction buffer (Fig. 1, Lane 3 & 4 vs. Lane 5). However, the reaction was triggered by $MgCl_2$ (Figure1, Lane 1 vs. Lane 3 & 4). Provided over-quantity REs (10U per reaction), incubation time did not significantly affect the final results (Fig. 1, Lane 3 vs. Lane 4). Moreover, compared with other REs, EcoR I showed star activity in PCR buffer with Mg^{2+} supplemented (Fig. 2, Panel B, Lane 4–7)

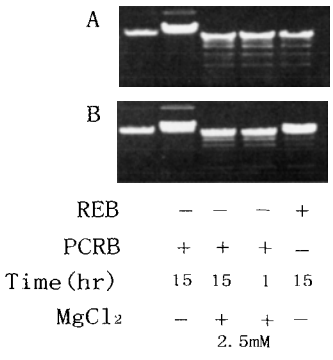

Fig. 1 Representative digests of lambda DNA by Ban II (A) and Sca I (B) in various buffers.

The results of other REs were not shown. In each panel, 1 contained undigested lambda DNA; Lane 2: lambda DNA digested in PCR buffer (PCRB) without Mg^{2+} supplemented; Lane 3 and Lane 4: digestion of lambda DNA with indicated incubation time in PCR Buffer (with a 1X composition of 50 mmol·L^{-1} KCl, 10 mmol·L^{-1} Tris-HCl, 0.1% Triton X-100) with 2.5 mmol·L^{-1} Mg^{2+} supplemented; Lane 5: digestion of lambda DNA in the recommended Restriction Enzyme Buffer (REB) provided with each enzyme. The results demonstrated $MgCl_2$ acted as an activator for activity of REs in PCRB.

Further experiments demonstrated various concentration of Mg^{2+} did not significantly affect the digestive activity of each RE (see Figure 2). Moreover the star activity of EcoR I seemed to be unrelated to concentration of $MgCl_2$ (Panel B, Lane 4–7).

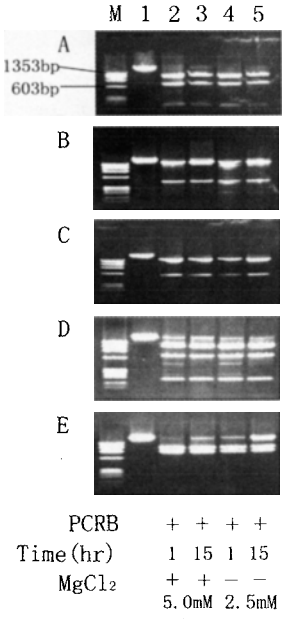

Fig. 2 Digests of lambda DNA in various buffers with different concentration of $MgCl_2$

The reaction system and conditions were the same as those indicated in Fig. 1, except that all tubes had been incubated for 15 h and reactions in Lane 3–7 contained 0, 2.5 mmol·L^{-1}, 5 mmol·L^{-1}, 7.5mmol·L^{-1} and 10 mmol·L^{-1} $MgCl_2$, respectively. Panel A was for Ban II; B, EcoR I; C, Hind III; D, Sac I; and E, Sca I. In each Panel, Lanes 1 contained undigested lambda DNA; Lane 2: digestion of lambda DNA in the recommended Restriction Enzyme Buffer (REB) provided with each enzyme; Lane 3–7: digestion of lambda DNA in PCR Buffer (with a 1X composition of 50 mmol·L^{-1} KCl, 10 mmol·L^{-1} Tris-HCl, 0.1% Triton X-100) with indicated final concentrations of Mg^{2+} above. Note that the concentrations of $MgCl_2$ do not affect activity of each RE.

All REs worked well for PCR products digestion (Fig. 3), even EcoR I did not show any star activity any more (Fig. 3, Panel B, Lane 2–5). The digests were the same as predicted.

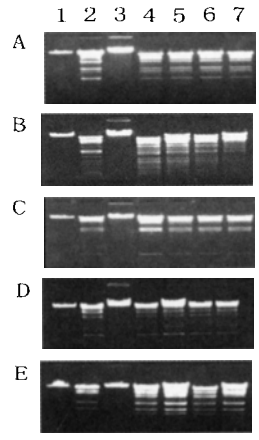

Fig. 3 PCR products directly digested with restriction enzymes in PCR buffer.

The panels were arranged as the same order of REs as those of Fig. 2. For each RE, predicted fragment sizes (bp) were given in parentheses after each enzyme. Panel A, Ban II (1105, 674, 151); Panel B, EcoR I (1483, 447); Panel C, Hind III (1394, 536); Panel D, Sac I (1105, 674, 151); Panel E, Sca I (1064, 866). For each panel, Lanes M, molecular markers ΦX174/Hae III; Lane 1: undigested PCR product; Lane 2 and 3: digestion of lambda DNA in PCR Buffer with additional Mg^{2+} supplemented, and final concentration of $MgCl_2$ arrived 5 mmol·L^{-1}; Lane 4 and 5: digestion of PCR products in PCR Buffer without additional Mg^{2+} supplemented as indicated incubation times. Note that the star activity of EcoR I disappeared in Panel B.

Discussions

Usually specific recognizing sites of no more than 15 REs were incorporated into the multiple cloning sites (MCS) region of any commercial vector. We chose five mostly commonly used REs, bought separately from different companies, to directly digest unmethylated lambda DNA. Whether one RE function normally or not was measured by comparing the resulting restriction pattern with the pattern obtained using the recommended restriction enzyme buffer. The results of both above indicated most REs functioned well, though EcoR I appeared star activity during digesting lambda DNA. All enzymes we used required addition of magnesium to the PCR Buffer to facilitate digestions, which proved that $MgCl_2$ is trigger activator for normal function of REs within the PCR buffer. Meanwhile the results suggested a wild range of magnesium concentration (2.5 to 10 mmol·L^{-1}) be suitable for complete function of REs tested. This feature would be helpful for PCR products digestion since partial Mg^{2+} had been consumed by the PCR components as polymerase, dNTPs and primers, etc.; if more magnesium added after amplification, it would not effect complete function of following REs digestion.

We further were interested in REs directly digesting PCR products in PCR buffer to determine the possible roles of other factors (e.g., the presence of affecting RE activity. The 1.9kb DNA fragment generated by PCR being digested exactly as predicted from DNA sequence, indicated other components (dNTPs, *Taq* DNA polymerase or oligonucleotide primers) within PCR Buffer do not significantly affect the activities or specificities of the REs. Direct comparison of the PCR Buffer with the typical REB shows wide variation in the components (Table 1), particularly with respect to pH, Tris-HCl concentration, magnesium concentration, and seems to provide no useful information to predict function of REs in PCR buffer. This implies many REs are able to retain their specificity and a significant degree of

activity despite a wide variation in buffer composition. Of course, the behavior of EcoR I within two kinds of experiments surprised us, we guess very possibly is it due to changes of pH prior to and after PCR amplification. This issue still needs further data.

For each digestion, ten units of RE used was a commonly recommended quantity for routine laboratory utilization (Sambrook *et al.* 2001), we also tested three units of RE for each reaction (results unshown), the final results were same. In some digestion reactions, a partial digest after 1 h or 15 h (e.g. Fig. 3, Panel D, Lane 2-5) was not considered a problem because this could be readily overcome with the addition of more each enzyme or by performing longer time digestion.

Table 1. Recommended Working Conditions by Manufacturers of Each REs

Enzyme	Tris ($mmol \cdot L^{-1}$)	$MgCl_2$ ($mmol \cdot L^{-1}$)	pH	Star Activity
Ban II	10	7	7.5	no
EcoR I	100	10	7.5	yes
Hind III	50	10	8.0	yes
Sac I	10	10	7.0	yes
Sca I	10	6	7.4	yes

We also tested Acc I (data not shown), which showed similar results and functioned normally within PCR buffer. Even only six REs tested, this research shows PCR products may be readily digested directly in the PCR tube immediately after amplification, which eliminates the requirement for expensive and tedious purification and/or precipitation steps to ensure that the amplified DNA is re-suspended in an appropriate REB. This simplified method for RE digestion of PCR products will have applications in RFLP analysis and SSCP analysis of large PCR products. However, it still needs further experiments to address this procedure being suitable for cloning applications.

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